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Effects of Sex-Ratio (SR)-Spiroplasma Infection on *Drosophila* Primary Embryonic Cultured Cells and on Embryogenesis

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ABSTRACT—Effects of Sex-ratio(SR)-spiroplasma infection in *Drosophila melanogaster* were examined in two experiments, *in vitro* and *in vivo*. The NSRO strain of the SR-spiroplasmas, transovarially transmitted microorganisms causing male-specific lethality in embryos, was collected from adult female hemolymph and inoculated into primary embryonic cell cultures prepared 2 or 5 days earlier. Within 1–2 days necrotic cell masses were detected in some neuroblastic and fibroblastic cells, but no detectable changes were observed in muscle cells, nerve cells, or in cellular spheres under a phase-contrast microscope. Spiroplasma-like structures were observed most abundantly in intercellular spaces of necrotic cells under the electron microscope. Similar experiments with NSRO-A, a non-male killing variant derived from NSRO, produced no such necrotic changes in neuroblastic and fibroblastic cells. Effects of NSRO on embryogenesis were examined under electron microscopy on embryos produced from NSRO-infected mothers. In about one-half the cases (presumably males) of embryos, 7.5–8.5 hr after oviposition, extensive necrotic cell masses were observed at regular intervals along the ventral side. In the remaining one-half (presumably females) of the embryos and in embryos produced from non-infected mothers, necrotic cell masses at regular intervals were also observed, but in a very much reduced scale, representing most probably the normal programmed cell death.

INTRODUCTION

The sex-ratio organisms (SROs), transovarially transmitted spiroplasmas (Order Mycoplasmatales) [1] (originally referred to as the sex-ratio spirochetes), which infect *Drosophila* and cause male-specific lethality at the embryonic stages, have been studied rather extensively [review, 1]. The SROs were found initially to infect a fraction of natural populations of the four neotropical species, *D. willistoni, D. nebulosa, D. equinoxialis* and *D. paulistorum*. Since, however, these microorganisms are found most abundantly in the hemolymph of adult females and can be transferred by microinjection into other *Drosophila* species where they establish permanent infection and show male-specific lethality, most studies have been carried out using *D. melanogaster* as a new host where various mutations and techniques to manipulate chromosomes are available.

Earlier studies have established that the infection of SROs results in the lethality of only the single-X individuals regardless of their phenotypic sex [2, 3, 4, 5], and the single-X diploid cells but not polyploid or polynucleated cells in primary embryonic cell cultures prepared from SRO-infected mothers [6]. Analysis of gynandromorph survivors from the SRO-infected mothers suggested the primary site of the lethal action of SRO included most of the primordial nervous and mesodermal tissues [7]. Only recently, however, the transmission process of the SRO into oocytes during oogenesis was described at the ultrastructural level [8], and morphological features of development in embryos infected with SRO were

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given [9]. We report here (1) that the infection of the SRO (NSRO strain) to the primary embryonic cell cultures prepared from Oregon-R strain of *D. melanogaster* produced characteristic necrotic cell masses in neuroblastic and fibroblast-like cells, and (2) that during embryogenesis, in 7.5–8.5 hr-embryos derived from NSRO-infected mothers some characteristic necrotic masses which distributed at regular intervals along the ventral side were detected.

MATERIALS AND METHODS

SRO strains

The NSRO (male-killing) and NSRO-A (non-male-killing) strains were used. The NSRO, derived originally from *D. nebulosa,* had been transferred to and maintained in Oregon-R strain of *D. melanogaster* (ORNSRO). The NSRO-A is a variant which appeared spontaneously among progenies of ORNSRO [10]. Fly stocks were maintained as described previously [11].

Since NSRO and NSRO-A had not been cultivated *in vitro,* samples containing each of these microorganisms were prepared as follows. ORNSRO or ORNSRO-A females aged 7–10 days were first injected with 0.15 M NaCl solution as much as possible, and within a few min the hemolymph (SRO is found most abundantly in the adult hemolymph) was collected by using glass microinjection pipettes. Two hundred μl each of such samples, each from 300–600 females, was then diluted with the same volume of 0.15 M NaCl solution, filtered through a Millipore filter (pore size, 0.45 μm), checked for the presence of the SRO under a dark-field microscope, and then used to infect the primary embryonic cell cultures.

The hemolymph of non-infected Oregon-R similarly prepared and checked for the absence of the SRO was also used as a control.

Embryonic cell culture

The primary cultures of *Drosophila* embryonic cells were prepared as described previously [12, 13]. Newly-laid eggs were collected from the wildtype Oregon-R strain of *D. melanogaster.* The eggs were dechorionated by treatment with 3% sodium hypochloride solution for 6 min, and then washed with distilled water, and transferred to physiological salt solution (0.7 g NaCl, 0.02 g KCl, 0.002 g $CaCl_2 \cdot 2H_2O$, 0.01 g $MgCl_2 \cdot 6H_2O$, 0.05 g $NaHCO_3$, 0.02 g $NaH_2PO_4 \cdot 2H_2O$ and 0.08 g glucose in 100 ml of distilled water) and allowed to develop at 25°C. The developmental stages of dechorionated eggs were readily determined through the transparent vitelline membrane under a binocular microscope.

Embryos at the postgastrula stage were selected, sterilized in 70% ethyl alcohol for 10 min, and washed three times with the sterile physiological salt solution. One hundred embryos were transferred to culture medium in a hollow slide and torn into small fragments with a pair of fine needles under a binocular microscope. Then, small torn fragments of tissues and cells were transferred into culture medium on a carbon-coated coverslip in a T-5 culture flask. The flasks were closed with a stopper and incubated at 25°C.

The culture medium consisted of medium K-17 of Kuroda [12], supplemented with 0.1 mg/ml fetuin (Grand Island Biol. Co., Deutsch Method) and 15% fetal bovine serum (Microbiol. Assoc. Inc., U.S.A.).

Infection of SR-spiroplasmas

The primary cultures of embryonic cells prepared as described above were incubated at 25°C. After incubation for 24 hr, almost all tissue fragments and cells were adhered on a glass surface of the culture flasks. Some characteristic types of cells were observed in cultures.

Upon further cultivation for a few days, muscle cells, epithelial cells, fibroblastic cells, nerve cells and cellular spheres were identified by their characteristic morphology and movement under a binocular microscope [12, 13].

After incubation for 2 or 5 dyas at 25°C, the primary cultures of embryonic cells were infected with 100 μl of the sample of SR-spiroplasmas. The cultures were incubated for 2 days at 25°C and observed every day under a phase-contrast microscope and photographed.

Electron microscopy

Cultured cells on cover slips were prefixed in

2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3. Whole embryos, collected at intervals and incubated for various periods of time, were dechorionated and punctured with a fine tungsten needle in a prefix solution (3% glutaraldehyde, 1% paraformaldehyde, 1% DMSO in 0.1 M cacodylate buffer at pH 7.2). Both materials were then postfixed in 1% OsO_4 in 0.1 M cacodylate buffer at pH 7.3, dehydrated in an ascending ethanol series, and embedded in Epon 812. Semi-thin sections cut with an LKB ultrotome were stained with Toluidine blue. Thin sections were stained with uranyl acetate and lead citrate and were examined with a Hitachi H-700 or a JEM 1200 EXII electron-microscope operated at 80–175 kV.

RESULTS

Effects of NSRO on embryonic cells in culture

When cells obtained from *Drosophila* embryos at the post-gastrula stage were cultured at 25°C, various types of cells differentiated after a few days. Among them, spindle-shaped muscle cells, flat polygonal epithelial cells, fibroblastic cells, baloon-like cellular spheres and nerve cells with many stretching and branching nerve fibers were conspicuous in their specific morphology and movement [12, 13]. When cultures were infected with male-killing NSRO after cultivation for 2 or 5 days and incubated for one or two more days, some brown and black necrotic cells appeared in nerve cell masses (Figs. 1a and 1b). Although these necrotic cells were not identified definitely, they had some characters of neuroblastic or fibroblastic cells in the site of their presence and their morphology. Cellular spheres, many spindle shaped muscle cells and hemocytes, which were assumed to be derived from the same embryonic tissue fragments and observed around the nerve cell masses, were not affected by infection of NSROs and showed normal morphology (Figs. 1c and 1d). In the preparation of the cultures, cells from 100 embryos were co-cultured together in the same flask. Cells from male and female embryos thus contributed 50% each in culture. Many neuroblastic and fibroblastic cells showed normal morphology in the culture. It may be assumed that the normal cells are derived from female embryos, and the necrotic cells are derived from male embryos.

When the primary cultures of Oregon-R embryonic cells were infected with non-male-killing mutant spiroplasmas, NSRO-A, necrotic cells such as those appeared in NSRO-infected cells were only very infrequenctly detected (Figs. 1c and 1d). This indicates that NSRO-A spiroplasma had no deleterious effects on embryonic cells of *Drosophila* cultured *in vitro*.

When hemolymph obtained from non-infected Oregon-R adult females was added to the primary cultures of embryonic cells, cells and tissues showed no detectable changes in their morphology upon further cultivation.

The cell cultures were processed for electron microscopic examinations. Structures identical to the SRO as previously described [8] were detected in intercellular spaces of cells in the primary cultures infected with NSRO often in close proximity to necrotic cells (Fig. 2a). Similar structures were also detected in cultured cells infected with NSRO-A (Fig. 2b). It was noted that the density of NSRO in such intercellular spaces of cells infected with NSRO was always higher than that in cells infected with NSRO-A. It is suggested that these observations on the presence of SRO-like structures may correspond to the presence of necrotic cells.

Effects of NSRO on cells in embryogenesis

NSRO-infected females from the stock cultures kept for many generations were mated to normal males and allowed to lay eggs. Eggs were collected at 60-min intervals and allowed to develop for 3.5–4.5, 7.5–8.5 and 12–13 hr, and processed for electron microscopy. Embryos at the early stage of 3.5–4.5 hr after oviposition showed no noticeable changes in cells and tissues compared with those in embryos produced from non-infected females. Some embryos, at the stage of 12–13 hr, produced badly-preserved features, while others at this stage were quite normal: the former seemed to be the NSRO-affected dying male embryos. Embryos at the stage of 7.5–8.5 hr, on the other hand, showed some characteristic features. Three out of 8 embryos at this stage examined showed the pre-

sence of many necrotic cell masses extensively spread along the ventral side at regular intervals (Fig. 3a). Three others showed similar but less extensive necrotic cell masses (Fig. 3b). In the remaining two embryos, necrotic cell masses were detected only scarecely. Since necrotic cell masses detected with similar features of the second and the last classes were also observed in normal non-infected wild type embryos (Fig. 3c), they are considered to represent normal programmed cell death. Most probably, then, the first class of the necrotic cell masses may occur in NSRO-infected

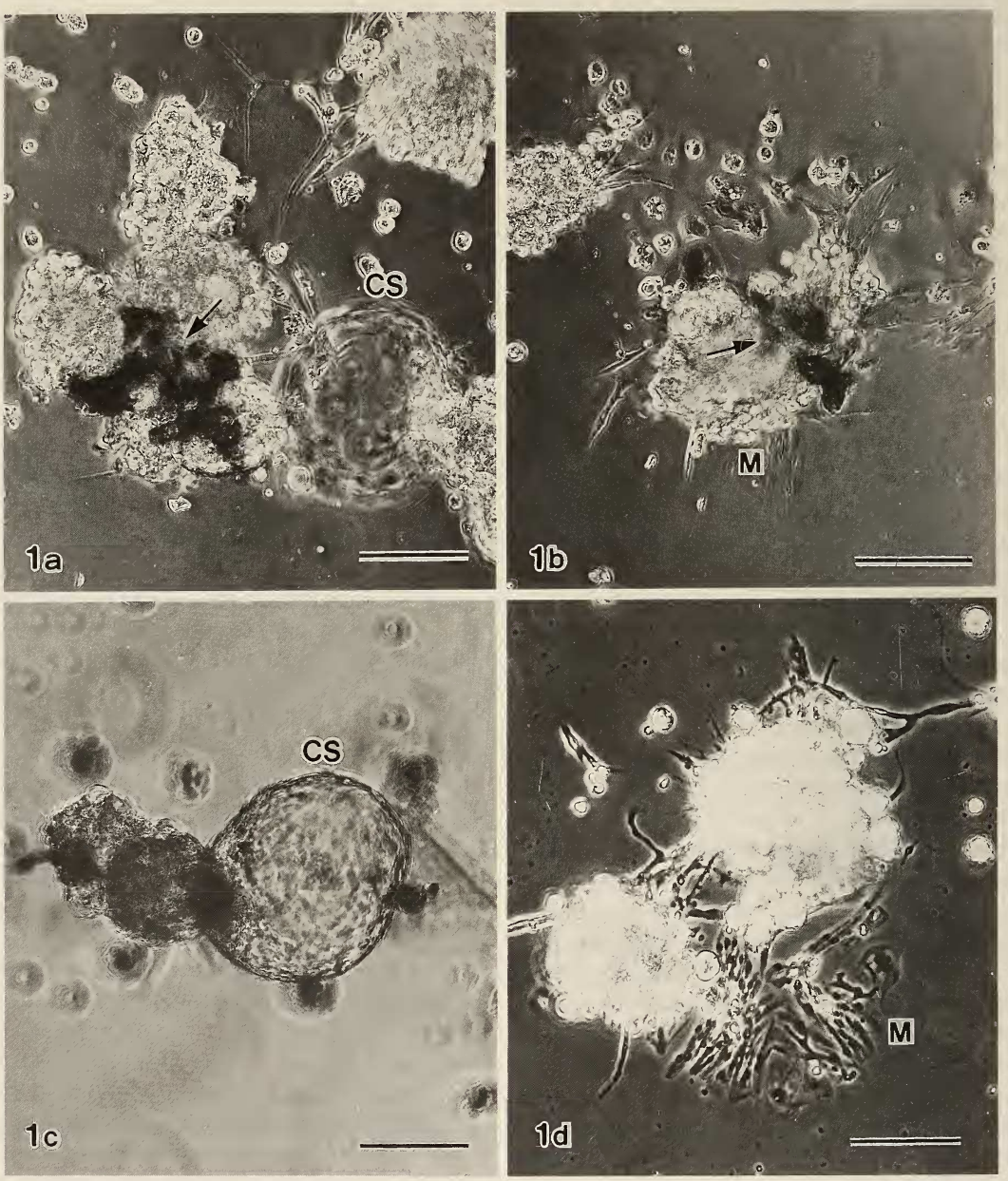


FIG. 1. Phase-contrast photomicrographs of part of embryonic cells cultured for 1 day and then infected with the male-killing NSRO (a, b), and of those infected with non-male-killing NSRO-A (c, d). Arrows indicate necrotic cells in the nerve cell mass. A cellular sphere (CS) and muscle cells (M) are not affected. Scales indicate 50 μm.

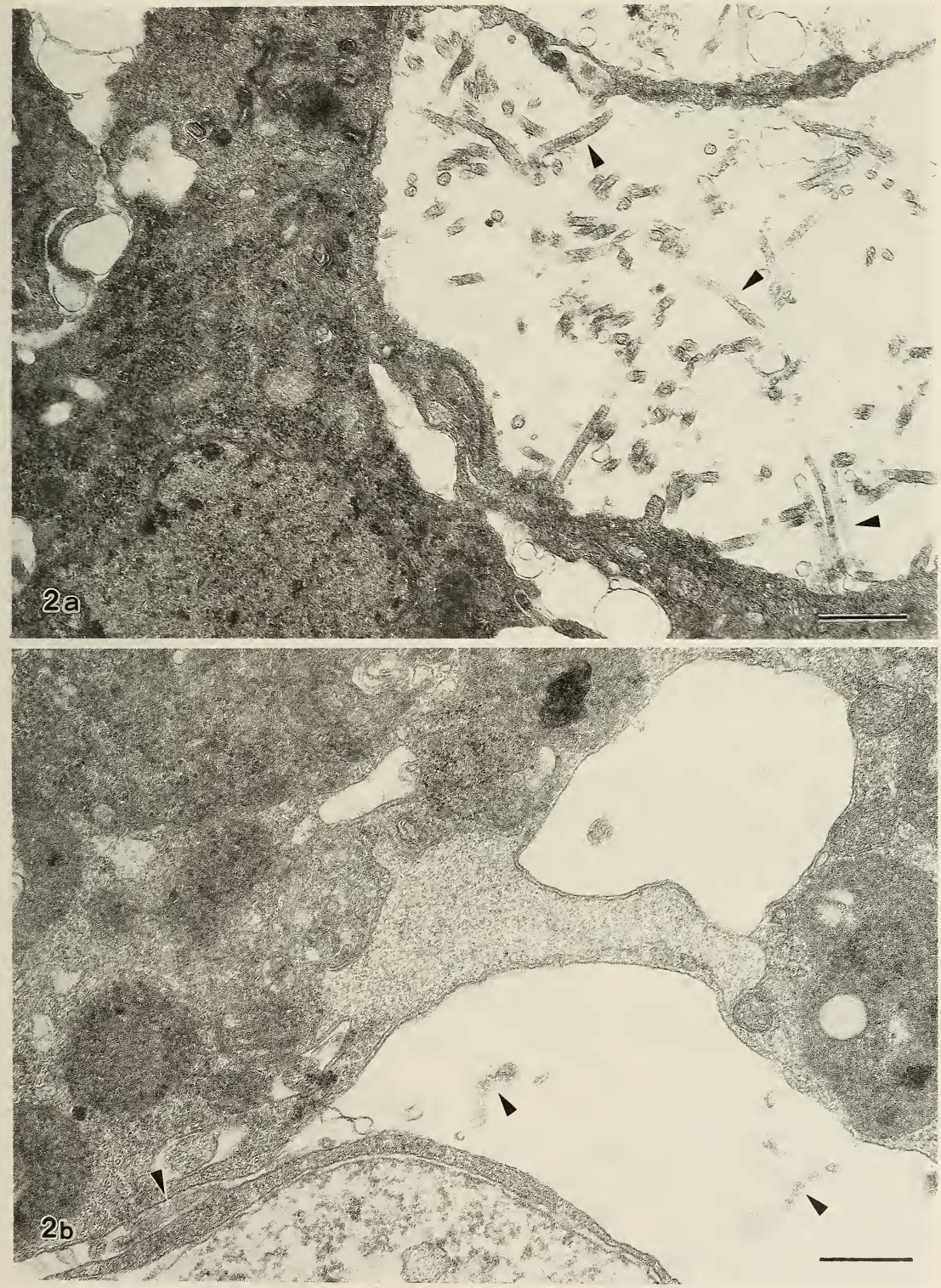
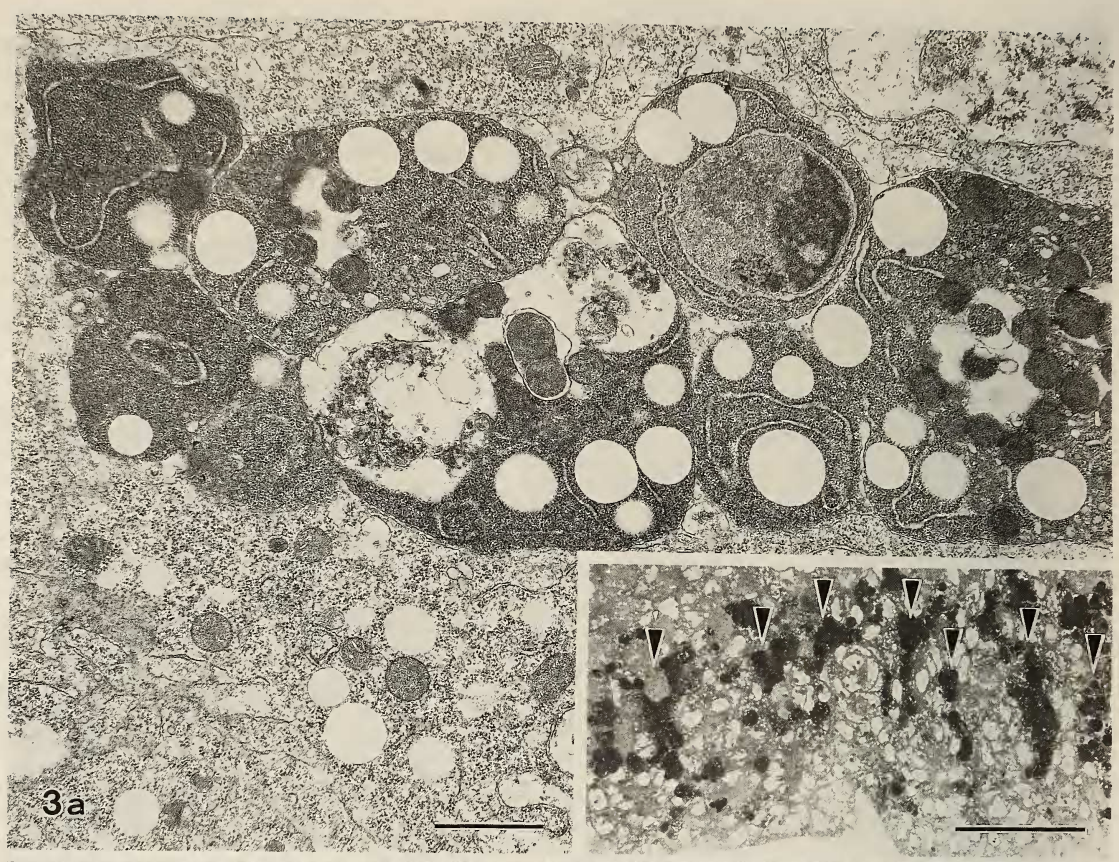
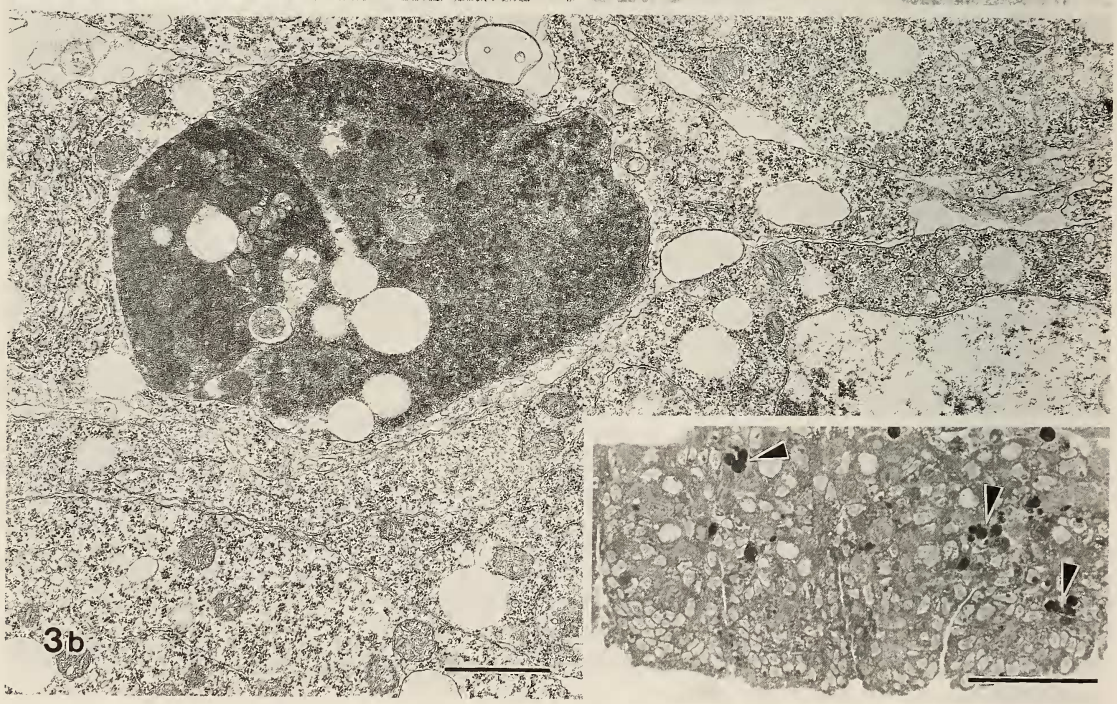


FIG. 2. Electron micrographs of part of embryonic cells cultured for 1 day and then infected with NSRO (a), and of those infected with NSRO-A (b). SRO-like structures (arrowheads) are seen. Scales indicate 1 μm.


3a


3b

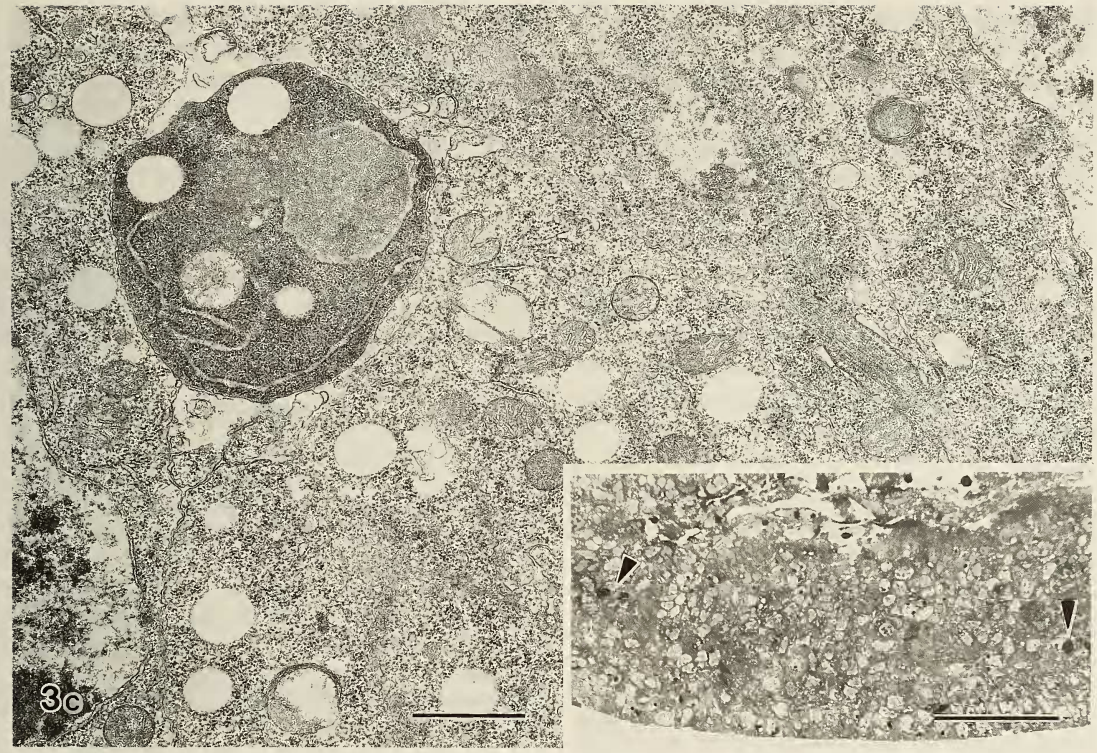


FIG. 3. Electron micrographs of thin sections and photomicrographs (inset) of semi-thin sections of embryos at stages of 7.5–8.5 hr after oviposition from an NSRO-infected mother fly (a, b), and from non-infected control mother fly (c). Necrotic cell masses appear dark in both light (arrowheads) and electron micrographs. Scales indicate 1 μm in the electron micrographs and 10 μm in the light micrographs.

male embryos, while the latter two classes of the necrotic cell masses may occur in the female embryos. Based on the positions and the morphological features of these necrotic cells, it is suggested that they are neuroblastic cells.

DISCUSSION

It was previously established that SROs kill selectively only male embryos of *Drosophila*, but do not affect female embryos (for a review see [1]). However, it is not clear how the SR spiroplasma recognizes the difference in male and female embryos, and what types of cells or tissues of male embryos are affected and killed by the SR spiroplasma. Study on the effects of SRO infection on the viability of gynandromorphs of *D. melanogaster* suggested that the primary site of action of SRO included the primordial nervous and mesodermal tissues [7].

In the electron microscopic study [8], it was found that, during oogenesis, SROs were transmitted into oocytes through a tunica propria, a noncellular membrane surrounding the egg chamber, and moved toward oocytes passing through the follicle cell layer. They were incorporated into ooplasm by pinocytosis and infolded in intracellular vesicles and yolk granules [8]. In the embryogenesis of the SRO-infected strain, it was found that abnormality occurred in the ventral nervous system [9]. The occurrence of necrotic, seemingly degenerating cells in cluster, was found in the ventral nervous system.

In the cell cultures of single embryos obtained from SRO-infected females, it was observed, under a phase-contrast microscope, that neurons, imaginal disc cells and plasmatocyte-like cells barely differentiated [6]. In the present experi-

ment, more direct evidence that the primary target cells of SROs may be nerve cells was obtained using the primary cultures of embryonic cells infected *in vitro* with the SRO. The SRO-infected cells showed necrotic changes in fragments of the nervous tissue. Other cell types such as muscle cells, epithelial cells, hemocytes and cellular spheres derived from the same embryos, were not affected by infection with SRO. Electron microscopic examinations confirm these observations that necrotic cells are neuroblastic cells. In the primary culture cells infected with SRO *in vitro*. SRO-like structures were detected in intercellular spaces under the electron microscope. The accumulation of SRO-like structures in intercellular spaces suggested that the target cells of SRO may produce some attracting factor(s) to SRO. Another possibility, although not mutually exclusive, may be that SRO has a high activity to proliferate only in such spaces under the *in vitro* culture conditions. It was reported that SRO showed a transient proliferation more than 100 times the initial concentration in primary *Drosophila* embryonic cell culture [14]. Accumulation of SRO in particular intercellular spaces may be one of the initial steps of *in vitro* proliferation of SRO.

Another interesting finding in the present study is the presence of many necrotic cell masses extensively spread along the ventral side at regular intervals. These necrotic cells may be neuroblastic cells, on the basis of their position in the embryo and their morphology. Tsuchiyama-Omura *et al.* [9] reported the presence of necrotic cells in the NSRO-infected 5–6 hr-old and older embryos. However, they did not observe extensive necrotic cell masses at regular intervals along the ventral side as described in this communication. The discrepancy between these observations remains to be clarified. They found necrotic cells in the disorganized mid-ventral portion of the midgut and also, in greater concentrations, inside the yolk mass of the NSRO-infected 10 hr-old embryos. It is possible that necrotic cell masses are "removed" rapidly from their original places and are "discarded" into the yolk mass. Slight changes in staging the embryonic age may then give quite different figures. In any case detalied electron microscopic examinations are required and with warrant, especially since our observations also suggested the presence of normal programmed cell death.

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